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Molecular Genetic Evidence that Dinoflagellates Belonging to the Genus *Symbiodinium* Freudenthal Are Haploid

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Abstract. Microscopic and cytological evidence suggest that many dinoflagellates possess a haploid nuclear phase. However, the ploidy of a number of dinoflagellates remains unknown, and molecular genetic support for haploidy in this group has been lacking. To elucidate the ploidy of symbiotic dinoflagellates belonging to the genus *Symbiodinium,* we used five polymorphic microsatellites to examine populations harbored by the Caribbean gorgonians *Plexaura kuna* and *Pseudopterogorgia elisabethae;* we also studied a series of *Symbiodinium* cultures. In 690 out of 728 *Symbiodinium* samples *in hospite* (95% of the cases) and in all 45 *Symbiodinium* cultures, only a single allele was recovered per locus. Statistical testing of the *Symbiodinium* populations harbored by *P. elisabethae* revealed that the observed genotype frequencies deviate significantly from those expected under Hardy-Weinberg equilibrium. Taken together, our results confirm that, in the vegetative life stage, members of *Symbiodinium,* both cultured and *in hospite,* are haploid. Furthermore, based on the phylogenetics of the dinoflagellates, haploidy in vegetative cells appears to be an ancestral trait that extends to all 2000 extant species of these important unicellular protists.

Introduction

The ploidy of an organism can significantly affect genome evolution. For example, diploids carry twice as much DNA as haploids and may be expected to accumulate new beneficial mutations at a higher rate (Paquin and Adams, 1983). The genome of diploids may also evolve rapidly because they carry more than a single copy of an allele. These "extra" alleles, over time, may evolve new functions while "old" alleles continue to perform their original functions (Lewis and Wolpert, 1979). Haploids, on the other hand, tend to have deleterious mutations purged more rapidly from the population since they are not masked (Hughes and Otto, 1999). Furthermore, knowledge of ploidy is essential to the interpretation and understanding of population genetic data. Given the importance of ploidy to genome evolution and population genetics, it is surprising that questions pertaining to it remain for a number of organisms.

Dinoflagellates are a diverse and ecologically important group of unicellular protists. For example, some species are major photosynthetic or heterotrophic components of the plankton, and others are considered to be the causative agents of fish kills. Microscopic and cytological evidence from the species examined to date suggests that dinoflagellates (with the exception of *Noctiluca* spp.) possess a vegetative haploid nuclear phase (Pfiester and Anderson, 1987; Coats, 2002). However, ploidy has not been explicitly determined for a number of dinoflagellates, including the important genus *Symbiodinium* Freudenthal (Taylor, 1974). Members of *Symbiodinium,* commonly referred to as zooxanthellae, are intra- or intercellular symbionts of marine invertebrates, including foraminiferans, sponges, cnidarians, and molluscs (Glynn, 1996). Blank (1987) reconstructed the nucleus of *Symbiodinium kawagutii* and found that the chromosomes of this dinoflagellate could not be paired either by size, appearance, or distribution. This cytological result led to the speculation that the vegetative (coccoid) cells of *Symbiodinium* may be haploid (Blank, 1987). To date, the molecular genetic data necessary to establish the ploidy of these symbiotic dinoflagellates, or

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any dinoflagellate, are lacking. We resolve this lingering question by using microsatellites to elucidate the ploidy of *Symbiodinium*.

Microsatellites are simple, tandemly repeated DNA sequences (reviewed in Chambers and MacAvoy, 2000) that are distributed abundantly in the genomes of virtually all organisms (Bennett, 2000). These single-locus, multiallelic, codominant segments of DNA are highly versatile and accessible markers for population genetic studies. However, microsatellites have also been successfully applied as tools in determining an organism's ploidy. For example, recovery of two alleles from a single locus in a single individual or isolate demonstrates a diploid nuclear phase. On the other hand, if a single allele is recovered from each of multiple loci, and the result is replicated over multiple individuals or isolates, the data suggest a haploid nuclear phase. This rationale has been applied to numerous organisms, including a parasitic protozoan, *Trypanosoma cruzi* (Oliveira *et al.*, 1998); two parasitic fungi, *Magnaporthe grisea* (Brondani *et al.*, 2000) and *Aspergillus fumigatus* (Bart-Delabesse *et al.*, 1998); a diatom, *Ditylum brightwellii* (Rynearson and Armbrust, 2000); a bryophyte, *Polytrichum formosum* (van der Velde *et al.*, 2001); and a false spider mite, *Brevipalpus phoenicis* (Weeks *et al.*, 2001). In this investigation, we apply the same strategy to members of the genus *Symbiodinium*. Populations of *Symbiodinium* harbored by the gorgonians *Plexaura kuna* and *Pseudopterogorgia elisabethae*, as well as a series of *Symbiodinium* cultures, were screened with five polymorphic microsatellites. The results confirm that members of *Symbiodinium* are haploid in the vegetative life stage.

Materials and Methods

Biological materials and nucleic acid isolation

Samples of *Plexaura kuna* were collected from colonies at depths of 1–7 m at 10 sites in the San Blas Islands, Republic of Panama (n = 142); two sites in the Florida Keys (n = 12); and three sites in the Bahamas (n = 6). For *Pseudopterogorgia elisabethae*, samples were collected from 575 colonies at depths of 10–27 m at 12 sites in the Bahamas (n = 43–50 per site). The 12 *P. elisabethae* sites were Sweetings Cay, Gorda Rock, Little San Salvador, Cat Island, Rum Cay, Hog Cay, and two sites each at Abacos, Eleuthera, and San Salvador Islands. Immediately after collection, samples were preserved in either 95% ethanol or salt-saturated DMSO (Seutin *et al.*, 1991) or were frozen in a liquid nitrogen vapor shipper. Total nucleic acids were extracted and quantified from branch pieces (about 2 cm) of *P. kuna* according to the methods of Coffroth *et al.* (1992). Nucleic acids were extracted from branch pieces (about 3 cm) of *P. elisabethae* by first grinding the tissue in STE buffer (0.05 *M* Tris-HCl (pH = 8.0), 0.1 *M* EDTA, 0.1 *M* NaCl, 0.2% SDS) followed by a modified extraction protocol using the Prep-A-Gene DNA Extraction Kit (Bio-Rad Laboratories, Hercules, CA) (Shearer and Coffroth, unpubl.). These extraction methods extracted nucleic acids from both the gorgonian hosts and from their *Symbiodinium* populations *in hospite*.

Symbiodinium cultures, isolated from a range of invertebrate hosts and geographic locations (Table 1), were maintained as described in Santos *et al.* (2001). Total nucleic acids were extracted and quantified from fresh algal cultures (approximately 5×10^3 cells) as described above for the *P. kuna* samples. After extraction, all nucleic acid samples were stored at −20 °C.

Phylogenetic identification of Symbiodinium *populations and cultures*

The phylogenetic identity of the *Symbiodinium* populations harbored by *P. kuna* and *P. elisabethae*, as well as the *Symbiodinium* cultures, was determined by length heteroplasmy in domain V of chloroplast large subunit (cp23S) rDNA as described in Santos *et al.* (2003). This technique rapidly genotypes *Symbiodinium* isolates and places them into a phylogenetic framework based on cp23S-rDNA (Santos *et al.*, 2003).

Construction of Symbiodinium *microsatellite libraries*

Nucleic acids used in the construction of the microsatellite libraries for *P. kuna* and *P. elisabethae Symbiodinium* populations were obtained by different procedures. *Symbiodinium* was isolated from samples of *P. kuna* collected from around the Caribbean and purified of host tissue (described in Santos *et al.*, 2001) before the nucleic acids were extracted and quantified as described above for *P. kuna* samples. On the other hand, nucleic acids for the *Symbiodinium* microsatellite library from *P. elisabethae* were extracted from colonies collected at Sweetings Cay (n = 2) and San Salvador (n = 4) as described above. These preparations contained nucleic acids from both *P. elisabethae* and their *Symbiodinium* populations *in hospite*, with no effort being made to enrich for *Symbiodinium* nucleic acids. After extraction, nucleic acids were pooled according to host source and used to construct the microsatellite libraries. The libraries were constructed and screened for microsatellites with dinucleotide repeats as described in Ciofi and Bruford (1998).

Screening of microsatellite loci polymerase chain reaction primers

To ensure that the microsatellite loci were not part of the host gorgonian genome, PCR amplifications were carried out on nucleic acids extracted from planulae of *P. kuna* and *P. elisabethae*, which are asymbiotic upon release from the maternal colony (Kinzie, 1974; Coffroth *et al.*, 2001; see

Table 1

Information about the Symbiodinium *cultures that amplified with at least one of the two microsatellites primer sets designed for the* Symbiodinium *populations of* Pseudopterogorgia elisabethae

Host organism	Culture name	Collection location
Aiptasia pallida	FLAp#2	Florida Keys
A. pallida	FLAp#2 10AB	Florida Keys
A. pallida	FLAp#3 10AB	Florida Keys
A. pallida	FLAp#4 10AB	Florida Keys
Aiptasia pulchella	OkAp#1	Okinawa, Japan
A. pulchella	OkAp#9	Okinawa, Japan
A. pulchella	OkAp#10	Okinawa, Japan
Briareum asbestinum (polyp)[a]	#498[b]	Florida Keys
B. asbestinum (polyp)[a]	#579[b]	Florida Keys
B. asbestinum (polyp)[a]	#595[b]	Florida Keys
B. asbestinum (polyp)[a]	#1135[b]	Florida Keys
B. asbestinum (polyp)[a]	#1140[b]	Florida Keys
B. asbestinum (polyp)[a]	#1246[b]	Florida Keys
B. asbestinum (polyp)[a]	#1319[b]	Florida Keys
B. asbestinum (polyp)[a]	#1385[b]	Florida Keys
B. asbestinum (polyp)[a]	#1394[b]	Florida Keys
B. asbestinum (polyp)[a]	#1509[b]	Florida Keys
B. asbestinum (polyp)[a]	#1510[b]	Florida Keys
B. asbestinum (polyp)[a]	#1669[b]	Florida Keys
B. asbestinum (polyp)[a]	#1735[b]	Florida Keys
B. asbestinum (polyp)[a]	#1902[b]	Florida Keys
B. asbestinum (polyp)[a]	#2053[b]	Florida Keys
B. asbestinum (polyp)[a]	#2080[b]	Florida Keys
Plexaura kuna	Pk13	Florida Keys
P. kuna	Pk14	Florida Keys
P. kuna (polyp)[a]	Pk205	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk206[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk208[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk215	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk216	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk225[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk226[b]	San Blas Islands, Panama
P. kuna	Pk801	Florida Keys
P. kuna	Pk807	Florida Keys
P. kuna (polyp)[a]	Pk702[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk703[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk704[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk705[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk706[b]	San Blas Islands, Panama
P. kuna (polyp)[a]	Pk707[b]	San Blas Islands, Panama
Pocillopora damicornis	Pd	Hawaii
Porites evermanni	Pe	Hawaii
Pseudopterogorgia elisabethae	SSPe	Bahamas
Unknown anemone	Cane	Hawaii
Zoanthus pacificus	Zp	Hawaii

[a] A polyp is defined as a newly settled and metamorphosed planula.
[b] Cultures that were started from a single dinoflagellate cell.

below). Nucleic acids were extracted from the planulae as described above for samples of *P. kuna*. To test for the presence of template nucleic acids (*i.e.*, cnidarian host DNA), PCR amplifications were conducted using the universal n18S-rDNA primer set ss5 and ss3 (Rowan and Powers, 1991). The asymbiotic nature of the planulae was then confirmed by the absence of a PCR product when the zooxanthella-biased n18S-rDNA primers ss5 and ss3z were used (Rowan and Powers, 1991). Both sets of reactions were carried out under the conditions described by Rowan and Powers (1991). Details of the five *Symbiodinium* microsatellite loci, including the sequences of the PCR primers and GenBank accession numbers, are given in Table 2.

Microsatellite amplifications and allele detection for Symbiodinium *populations*

PCR reactions for each *Symbiodinium* microsatellite locus were performed in 10-μl volumes containing 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 0.001% gelatin, 200 μM dNTPs, 1 U *Taq* polymerase, approximately 10 ng of template DNA, and $MgCl_2$ and primers at concentrations detailed in Table 2. Thermocycling conditions were as follows: initial denaturing step of 94°C for 2–3 min, 35–39 cycles of 94°C for 30–45 s, annealing temperature of X° (see Table 2) for 30–45 s, 72°C for 30 s, and a final extension of 72°C for 3–5 min.

Most *Symbiodinium* microsatellite alleles from *P. kuna* were separated in 2% 0.5X Tris-borate (TBE) agarose gels and visualized by ethidium bromide staining. In addition, some *Symbiodinium* microsatellite alleles from *P. kuna* were amplified in the presence of ^{33}P-ATP (Sambrook *et al.*, 1989), separated in 6% polyacrylamide / 0.5X TBE gels under denaturing (7 *M* urea) conditions, and visualized by exposure to X-ray film. The sizes of *Symbiodinium* microsatellite alleles from *P. kuna* were determined either with a 100-bp DNA ladder (MBI Fermentas, Hanover, MD) for 2% agarose gels, or for polyacrylamide gels, with a ^{33}P-ATP puC19 plasmid sequencing reaction serving as a nucleotide size-ladder.

For *P. elisabethae*, *Symbiodinium* microsatellite alleles were labeled by incorporation of a 5′-IRD800 M13 Forward primer (see Table 2), and were separated in 25-cm-long, 0.25-mm-thick, 6.5% Long Ranger (FMC Bioproducts, Rockland, ME)/0.5X TBE gels under denaturing (7 *M* urea) conditions. Gel electrophoresis was performed at 1500 V, 40 W, 40 mA, 50 °C, and the default scan speed, with LI-COR's NEN Global IR2 DNA sequencer system (LI-COR Biotechnology Division, Lincoln, NE). The sizes of *Symbiodinium* microsatellite alleles from *P. elisabethae* were determined with the fragment analysis program Gene ImagIR v3.55 (Scanalytics Inc, Fairfax, VA) using DNA ladders (ladder 97–147 bp or 172–272 bp, rungs at 25-bp increments, ladders loaded every fifth lane) as size references.

In addition, *Symbiodinium* microsatellite-loci primer-sets for each host species were tested on the *Symbiodinium* populations of the other host species. For the host species

Table 2

Characterization and PCR amplification conditions of the five Symbiodinium *clade B microsatellite loci; size refers to the predicted PCR product size with original sequenced clone as template*

Microsatellite locus name	Motif type	Sequence of microsatellite motif	PCR primer sequences	Size (bp)
CA1′.7[a,b]	GT, interrupted pure	$(GT)_3GAT(GT)_4GC$ $(GT)_{13}GAT(GT)_2$	CA1′.7UP: 5′-GCAGTGTGTTACCTTAAAGTGCGG-3′ CA1′.7DN: 5′-GTTTAGTTGAGCAACTTCCGAG-3′	345
GA2.8[a,b]	CA, interrupted pure	$(CA)_7CG(CA)_{21}$	GA2.8UP: 5′-TCGACTCTTGGCGCAAAATG-3′ GA2.8DN: 5′-GGGATGAAACACTGGGATAATCCAG-3′	174
GA4.84[a,b]	CA, interrupted complex	$(CAGA)_2(CA)_8CGA$ $A(CA)_6(CG)_2CAGA$ $(CA)_{13}CG(CA)_5$ $(GA(CA)_2)_5GACG$ $(CA)_5$	GA4.84UP: 5′-GATCAAACTCTTGTGATGAC-3′ GA4.84DN: 5′-GTCAGATTGTCATCAAAGACTGC-3′	175
CA4.86[c,d]	CA, interrupted complex	$(CACC)CA$ $(CACC)_2(CA)_9$	CA4.86R: 5′-GCCTTCAATGCAATCACCTT-3′ CA4.86L[f]: 5′-GGAATTGGCCATCCCTCTAT-3′	193
CA6.38[c,e]	CA, interrupted pure	$(CA)_9CG(CA)_4$	CA6.38R: 5′-CAAAGAATATTCGGGGGTCA-3′ CA6.38L[f]: 5′-AGTTGATACGCCGGATGTGT-3′	112

[a] Microsatellite loci isolated from *Plexaura kuna Symbiodinium* populations *in hospite;* sequences deposited in GenBank under accession numbers AF549186-AF549188.

[b] 1.5 m*M* $MgCl_2$, 3 pmol of each primer, 60 °C annealing temperature.

[c] Microsatellite loci isolated from *Pseudopterogorgia elisabethae Symbiodinium* populations *in hospite;* sequences deposited in GenBank under accession numbers AF474165 and AF474169.

[d] 2.5 m*M* $MgCl_2$, 0.2 pmol CA4.86R, 0.18 pmol CA4.86LM13, 0.02 pmol 5′-IRD800 M13 Forward primer, 50 °C annealing temperature.

[e] 1.5 m*M* $MgCl_2$, 3 pmol CA6.38R, 2 pmol CA6.38LM13, 0.2 pmol 5′-IRD800 M13 Forward primer, 56 °C annealing temperature.

[f] Primers with M13 Forward sequence (5′-CACGACGTTGTAAAACGAC-3′) added for visualization on LI-COR's NEN® Global IR2 DNA Sequencer System (LI-COR Biotechnology Division, Lincoln, NE). See text for details.

comparisons, PCR reaction conditions and detection of *Symbiodinium* microsatellite alleles were as previously described.

A total of 45 *Symbiodinium* cultures were also screened with the five *Symbiodinium* microsatellite loci primer sets using the methods described above.

Statistical testing of microsatellite data

Exact probabilities (analogous to Fisher's exact test for 2×2 contingency tables) for the observed genotype frequencies and for those expected under Hardy-Weinberg equilibrium were used to determine whether populations of *Symbiodinium* harbored by *P. elisabethae* in the Bahamas deviate from Hardy-Weinberg equilibrium. Significance values were calculated with the computer program BIOSYS-2 (Swofford and Selander, 1981; modified by William C. Black, Department of Microbiology, Colorado State University; available at: ftp://lamar.colostate.edu/pub/web4/).

Results

Phylogenetic identification of Symbiodinium *populations and cultures*

The numerically dominant *Symbiodinium* in association with *Plexaura kuna* and *Pseudopterogorgia elisabethae* belong to *Symbiodinium* clade B (*sensu* Rowan and Powers, 1991), and more specifically, to *Symbiodinium* B184 (*sensu* Santos *et al.*, 2003). The *Symbiodinium* cultures also belong to clade B, with representatives from *Symbiodinium* B184, B211, and B223 (*sensu* Santos *et al.*, 2003; Table 3).

Genomic location of the microsatellite loci

Nucleic acids extracted from planulae of *P. kuna* and *P. elisabethae* failed to produce an amplicon with the zooxanthella-biased n18S-rDNA PCR primers ss5 and ss3z. On the other hand, an amplicon of approximately 1800 bp was generated from the same templates using the universal n18S-rDNA PCR primers ss5 and ss3 (data not shown). These results confirmed that the planulae were asymbiotic. Nucleic acids extracted from planulae and then used as templates for the microsatellite loci primer sets failed to produce PCR amplicons, whereas nucleic acids from adult tissue that contained *Symbiodinium* or nucleic acids isolated from *Symbiodinium* cultures produced PCR amplicons (see below) of the correct size (given in Table 2). Taken together, the data demonstrate that the microsatellite loci are located within the genome of *Symbiodinium*, and not that of the gorgonian hosts.

Table 3

Microsatellite and chloroplast genotypes for the Symbiodinium *cultures; a microsatellite genotype is defined as a unique combination of microsatellite allele sizes at loci CA4.86 and CA6.38*

Symbiodinium microsatellite allele size (bp)		*Symbiodinium* chloroplast large subunit rDNA (cp23S-rDNA) genotype[a]	Culture name	Host organism
Locus CA4.86	Locus CA6.38			
179	100	B184	Cane	Unknown anemone
		B184	OkAp#1	*Aiptasia pulchella*
		B184	OkAp#10	*A. pulchella*
		B184	OkAp#9	*A. pulchella*
		B184	Pd	*Pocillopora damicornis*
		B184	Pe	*Porites evermanni*
		B184	Zp	*Zoanthus pacificus*
179	102	B184	FLAp#2	*Aiptasia pallida*
		B184	FLAp#2 10AB	*A. pallida*
		B184	FLAp#3 10AB	*A. pallida*
		B184	FLAp#4 10AB	*A. pallida*
183	100	B184	Pk205	*Plexaura kuna* (polyp)[c]
		B184	Pk208[b]	*P. kuna* (polyp)[c]
		B184	Pk215	*P. kuna* (polyp)[c]
		B184	Pk216	*P. kuna* (polyp)[c]
		B184	Pk226[b]	*P. kuna* (polyp)[c]
187	"null"	B223	#498[b]	*Briareum asbestinum* (polyp)[c]
		B223	#579[b]	*B. asbestinum* (polyp)[c]
		B223	#1385[b]	*B. asbestinum* (polyp)[c]
191	104	B184	#595[b]	*B. asbestinum* (polyp)[c]
		B184	#1246[b]	*B. asbestinum* (polyp)[c]
		B184	#1394[b]	*B. asbestinum* (polyp)[c]
191	106	B184	#1135[b]	*B. asbestinum* (polyp)[c]
		B184	#1140[b]	*B. asbestinum* (polyp)[c]
		B184	#1319[b]	*B. asbestinum* (polyp)[c]
		B184	#1509[b]	*B. asbestinum* (polyp)[c]
		B184	#1510[b]	*B. asbestinum* (polyp)[c]
		B184	#1669[b]	*B. asbestinum* (polyp)[c]
		B184	#1735[b]	*B. asbestinum* (polyp)[c]
		B184	#1902[b]	*B. asbestinum* (polyp)[c]
		B184	#2053[b]	*B. asbestinum* (polyp)[c]
		B184	#2080[b]	*B. asbestinum* (polyp)[c]
		B184	Pk225[b]	*P. kuna* (polyp)[c]
		B184	Pk706[b]	*P. kuna* (polyp)[c]
		B184	Pk707[b]	*P. kuna* (polyp)[c]
193	98	B211	Pk702[b]	*P. kuna* (polyp)[c]
		B211	Pk703[b]	*P. kuna* (polyp)[c]
193	100	B184	Pk13	*P. kuna*
		B184	Pk14	*P. kuna*
		B184	Pk801	*P. kuna*
		B184	Pk807	*P. kuna*
193	104	B184	Pk206[b]	*P. kuna* (polyp)[c]
		B184	Pk704[b]	*P. kuna* (polyp)[c]
		B184	Pk705[b]	*P. kuna* (polyp)[c]
193	112	B184	SSPe	*Pseudopterogorgia elisabethae*

[a] *Sensu* Santos *et al.* (2003).

[b] Cultures that were started from a single dinoflagellate cell.

[c] A polyp is defined as a newly settled and metamorphosed planula.

Microsatellites of Symbiodinium *within a host species*

For the *Symbiodinium* populations of *P. kuna*, four unique alleles were identified at locus CA1′.7, three at GA2.8, and three at GA4.84. The sizes of the alleles at each locus were CA1′.7 (approximately 360, 380, 400, 420 bp), GA2.8 (approximately 180, 190, 200 bp), and GA4.84 (approximately 280, 300, 310 bp). In 147 cases, only a single

allele per locus could be detected from a *P. kuna* colony, but the *Symbiodinium* populations from 13 colonies produced two distinct alleles at one or more loci. Thus, 8.1% of the *Symbiodinium* populations sampled from *P. kuna* individuals produced more than a single allele at any microsatellite locus.

When the *Symbiodinium* populations of *P. elisabethae* were screened at loci CA4.86 and CA6.38, 8 and 10 unique alleles, respectively, were identified from 568 of the 575 colonies that were examined (nucleic acids from seven colonies failed to amplify at either locus and were excluded from further analysis). Allele sizes for loci CA4.86 and CA6.38 ranged between 185–207 bp and 96–122 bp, respectively (Fig. 1). In most of these cases, only a single allele per locus could be detected from the *Symbiodinium* population of a *P. elisabethae* colony (Fig. 2). In 25 cases (4.4%), the *Symbiodinium* population from a *P. elisabethae* colony possessed two distinct alleles at one or more loci (example in Fig. 2). This pattern is similar to that observed for *Symbiodinium* microsatellites isolated and amplified from *P. kuna*.

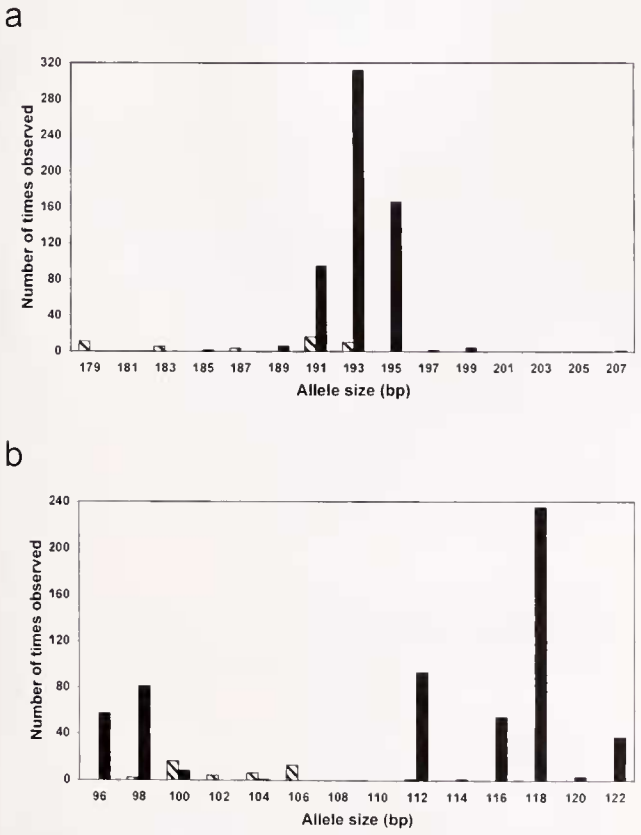

Figure 1. Size distribution of alleles at two microsatellite loci: (a) CA4.86 and (b) CA6.38. Solid bars, *Symbiodinium* populations of *Pseudopterogorgia elisabethae* (*in hospite*); diagonal strip bars, *Symbiodinium* cultures. Values for *P. elisabethae* are derived from all samples, include those that possessed two distinct alleles or a null allele at one or more loci.

Symbiodinium *microsatellites between host species*

Amplifications using the *Symbiodinium* microsatellite loci primer sets for one host species produced mixed results when applied to the *Symbiodinium* populations of the other host species. In many cases, the primers failed to produce an amplicon. However, when PCR amplification did occur, only a single allele was detected per locus (see fig. 4 of Santos *et al.*, 2001, for examples).

Microsatellites from Symbiodinium *cultures*

The *Symbiodinium* microsatellite loci CA1′.7, GA2.8, and GA4.84 were not detected in any of the *Symbiodinium* cultures. However, the 45 cultures did amplify with at least one of the two *Symbiodinium* microsatellites primer sets isolated from *P. elisabethae* (Table 2). At loci CA4.86 and CA6.38, 5 and 6 alleles, respectively, were identified (Table 3, Fig. 1). Allele sizes for loci CA4.86 and CA6.38 ranged between 179–193 bp and 98–112 bp, respectively (Fig. 1). In each case, however, only a single allele was detected per locus.

Test for deviations from Hardy-Weinberg equilibrium

The observed genotype frequencies of the *Symbiodinium* populations inhabiting *Pseudopterogorgia elisabethae* from most of the 12 sites in the Bahamas were significantly different from those expected under Hardy-Weinberg equilibrium (Table 4). An excess of "homozygote" (genotypes with a single allele per locus) genotypes was present in the populations compared to the expected number based on allelic frequencies. In four cases, a single monomorphic allele was recovered from the population (Table 4).

Discussion

Microsatellite loci isolated from members of *Symbiodinium* clade B were amplified by PCR to assess ploidy in these symbiotic dinoflagellates. From most *Symbiodinium* populations harbored by *Plexaura kuna* and *Pseudopterogorgia elisabethae* (690 out of 728 samples; 95% of the cases), only a single allele was recovered per locus, and all 45 *Symbiodinium* cultures possessed a single allele per locus. Furthermore, an excess of "homozygous," or single allele per locus, genotypes in the *Symbiodinium* populations of *P. elisabethae* violated Hardy-Weinberg equilibrium. Nonconformity to the prediction of Hardy-Weinberg equilibrium indicates that one or more of its assumptions are not met in the population. The assumptions include (1) the organism is diploid; (2) reproduction is sexual; (3) the population is infinitely large; (4) mating occurs randomly; (5) generations are nonoverlapping; and (6) the population is free of genetic drift, migration, mutation, and natural selection (reviewed in Hartl and Clark, 1989). The *Symbiodinium* populations of *P. elisabethae* may violate many of

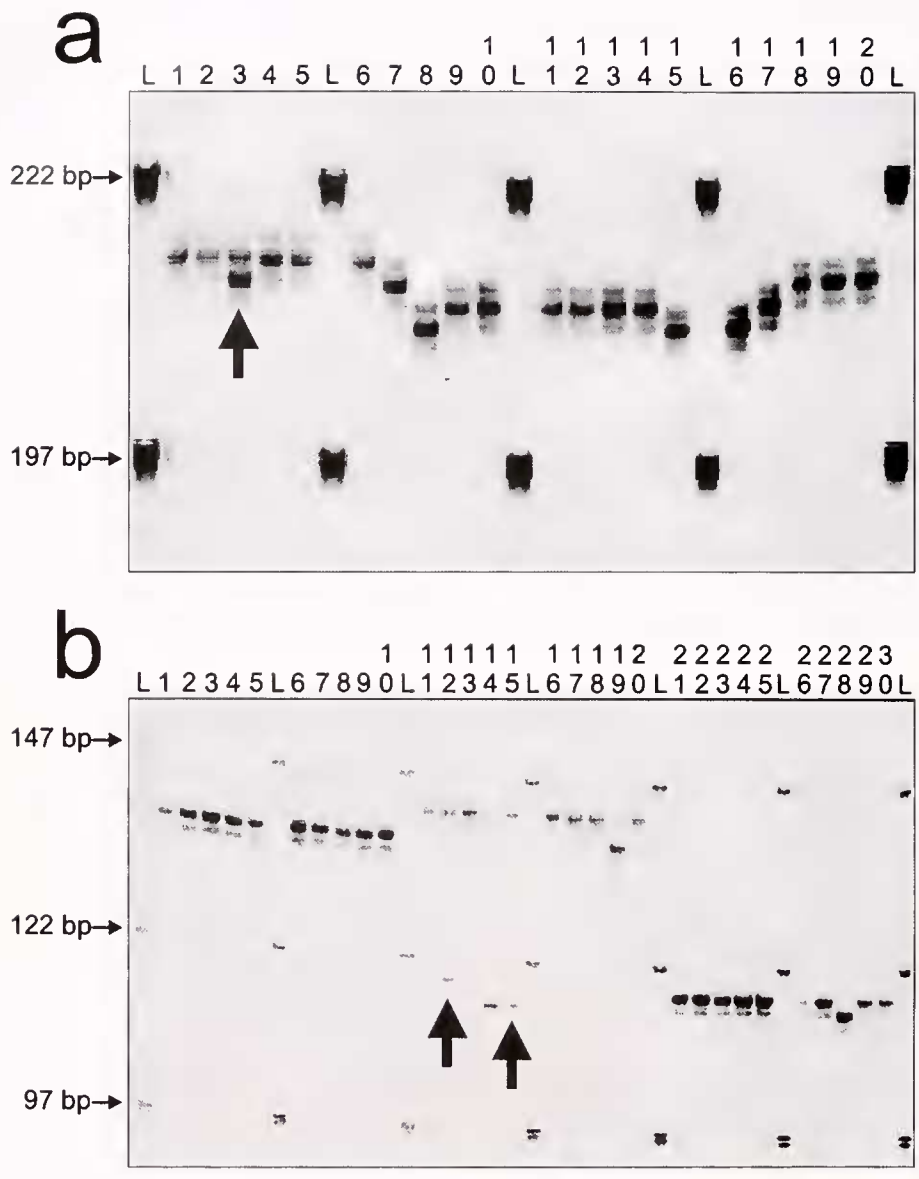

Figure 2. *Symbiodinium* populations from *Pseudopterogorgia elisabethae:* Polyacrylamide gel electrophoresis analysis of microsatellite alleles from two loci: (a) CA4.86 and (b) CA6.38. Number above lane represents individual samples of *P. elisabethae.* L represents DNA ladder lanes. Vertical arrows denote samples in which more than a single allele was amplified per locus.

these assumptions, but the predominance of "homozygous" genotypes in the *Symbiodinium* populations harbored by *P. kuna,* and in all *Symbiodinium* cultures, strongly suggests that at least the first assumption is being violated. If *Symbiodinium* were diploid in the vegetative life stage, more "heterozygous" genotypes should have been sampled. A possible explanation for the lack of heterozygous genotypes is that the *Symbiodinium* microsatellite loci are located in an organellar (*i.e.*, mitochondrial or chloroplast) genome. If so, a similar pattern of a single allele per locus would be observed. Although a microsatellite locus has been described from the chloroplast genome of a free-living dinoflagellate, *Heterocapsa triquetra* (Zhang *et al.,* 1999), it is highly unlikely that all five *Symbiodinium* microsatellite loci were isolated from the organellar genomes. Taken together, our results demonstrate that members of *Symbiodinium* clade B, both cultured and *in hospite,* possess a haploid nuclear phase. Moreover, since the genus is monophyletic (see, for example, Rowan and Powers, 1992; LaJeunesse, 2001; Santos *et al.,* 2002), our findings corroborate Blank's (1987) speculation that haploidy exists in *S. kawagutii* and extend it to all members of *Symbiodinium.*

If the vegetative life stage of *Symbiodinium* is haploid, why was more than a single allele sometimes recovered per

Table 4

Significant deviation of observed genotype frequencies from those expected under Hardy-Weinberg equilibrium for Symbiodinium *populations inhabiting* Pseudopterogorgia elisabethae *in the Bahamas*

Name/location of *Pseudopterogorgia elisabethae* populations (sample size)	*P* values	
	Locus CA4.86	Locus CA6.38
Sweetings Cay (44)	<0.001	<0.001
Gorda Rock (47)	<0.001	<0.001
Abacos Shallow (50)	<0.001	<0.001
Abacos Deep (48)	<0.001	<0.001
South Hampton Reef (47)	0.011	0.032
Little San Salvador (48)	<0.001	<0.001
East End Point of Eleuthera (44)	<0.001	<0.001
Cat Island (45)	<0.001	MA
San Salvador, Riding Rock (43)	MA	MA
San Salvador, Pillar Reef (50)	<0.001	MA
Rum Cay (46)	<0.001	<0.001
Hog Cay (46)	<0.001	<0.001

Values were calculated using exact probabilities (analogous to Fisher's exact test for 2 × 2 contingency tables) using the computer program BIOSYS-2 (Swofford and Selander, 1981; modified by William C. Black, Department of Microbiology, Colorado State University; available at: ftp://lamar.colostate.edu/pub/wcb4/). Samples that possessed a "null," or absent, allele at a locus were excluded from the analysis. MA designates a monomorphic allele recovered from the population.

locus *in hospite?* Among the samples of *P. kuna* and *P. elisabethae*, about 8.1% and 4.4%, respectively, gave this result. In these cases, the recovery of more than a single allele at a microsatellite locus suggests that the colonies harbored at least two genotypes of symbiotic algae. It has been demonstrated, using a combination of culturing and molecular techniques, that *P. kuna* and *P. elisabethae* can harbor more than a single genotype of *Symbiodinium* simultaneously (Goulet and Coffroth, 1997, 2003; Coffroth *et al.*, 2001; Santos *et al.*, 2001). Our data support this conclusion while suggesting that it is uncommon for colonies of these gorgonians to harbor more than one *Symbiodinium* genotype, at least at detectable concentrations. For these uncommon colonies, it remains to be determined whether the additional genotypes represent relicts of the initial symbiont uptake by the newly settled asymbiotic planulae or reflect populations that are secondarily acquired and contribute to the host in other ways.

Phylogenetic support for haploidy in Symbiodinium *and other dinoflagellates*

Our conclusion that *Symbiodinium* is haploid is consistent with data from most of the other dinoflagellates. The life cycle of nearly all dinoflagellates examined to date is dominated by asexual reproduction of haploid vegetative cells (Pfiester and Anderson, 1987). This observation, coupled with the monophyly of dinoflagellates (Saldarriaga *et al.*, 2001), suggests that all members of the dinoflagellates should possess a vegetative haploid nuclear phase. Interestingly, the vegetative cells of the red-tide dinoflagellate genus *Noctiluca* are thought to be diploid (Zingmark, 1970; Pfiester and Anderson, 1987). In *Noctiluca*, the first divisions of the gamete mother-cell nucleus are believed to be meiotic (Zingmark, 1970), which would imply diploidy in these dinoflagellates. Recently, the conclusion of diploidy in *Noctiluca* has been challenged (Schnepf and Drebes, 1993), but no definitive data have been presented to establish the ploidy of these dinoflagellates. We hypothesize, based on our knowledge of other dinoflagellates, that *Noctiluca* spp. possess a vegetative haploid nuclear phase. Analyses of microsatellite loci, such as we have done here for *Symbiodinium*, would be one way to test this hypothesis and settle the question of ploidy in *Noctiluca*.

A haploid nuclear phase in the dinoflagellates is consistent with that of their closest relatives. The Apicomplexa, obligate intracellular parasites of many vertebrate and invertebrate hosts, are thought to have evolved from, or shared a common ancestor with, the dinoflagellates (Wolters, 1991; Cavalier-Smith, 1993) about 395-929 Mya (Escalante and Ayala, 1995). The apicomplexan *Plasmodium falciparum*, one of the causative agents of human malaria, is haploid in its human host and only briefly diploid in its mosquito vector (Campbell, 1993; Conway *et al.*, 1999). Other apicomplexans, such as *Cryptosporidium parvum* and *Toxoplasma gondii*, also possess a haploid nuclear phase (Costa *et al.*, 1997; Feng *et al.*, 2002). Given the close evolutionary relationship between the two groups, the ancestral state in the progenitor of the apicomplexans and dinoflagellates was probably haploidy.

Evidence for fine-scale specificity in associations between host and Symbiodinium

Surprisingly, primer sets designed for amplification of the *Symbiodinium* microsatellite loci in one host species produced mixed results when applied to the *Symbiodinium* populations of the other host species or to *Symbiodinium* cultures. For example, primers designed for the symbiont populations of *P. kuna* were not very successful in amplifying the *Symbiodinium* populations of *P. elisabethae* colonies or algal cultures derived from a variety of hosts (Santos *et al.*, 2001; unpubl. data). Typically, the utility of a microsatellite system (*i.e.*, microsatellite primer sets) decreases as the phylogenetic distance between the samples being screened increases (Schlotterer, 1998). In these experiments, however, most (768 out of 773; 99.4%) of the samples belonged to a single lineage, *Symbiodinium* B184. Therefore, the microsatellite primer sets should have worked on all members of the group. This failure to amplify alleles from closely related, but non-focal, *Symbiodinium*

samples is probably due to mutational changes in the flanking regions of the microsatellite array. Mutations in these regions can lead to primer-template mismatch and thus to inhibition of the PCR reaction. In support of this hypothesis, we have sequenced alleles from loci CA4.86 and CA6.38 and found mutations, such as nucleotide substitutions and insertion-deletions (indels), in the microsatellite flanking regions from members of *Symbiodinium* B184, B211, and B223 (the evolution of *Symbiodinium* microsatellites will be discussed in a subsequent paper).

Although microsatellite alleles were not always recovered in the host species comparisons, an important conclusion can be drawn from these data. The specificity exhibited by the different microsatellite primer sets to the population of *Symbiodinium* from which they were designed and the consistent presence of "null," or absent, alleles in the other host species suggest that the two *Symbiodinium* B184 populations are genetically distinct from each another and specific to a given host species. The genetic differences are probably spread across the *Symbiodinium* genome, but at the minimum they are confined to mutations in the flanking regions of the microsatellite loci. Unfortunately, internal transcribed spacer (ITS) sequences—one of the most useful genetic markers for identifying *Symbiodinium* types (LaJeunesse, 2001)—are identical, or nearly so, in the two populations (Santos *et al.*, 2001); thus other genetic markers are needed to elucidate the relationship between them. Nevertheless, *P. kuna* and *P. elisabethae* appear to associate preferentially with genetically distinct *Symbiodinium* B184 populations, which provides evidence for fine-scale host-*Symbiodinium* specificity in these gorgonian species.

Microsatellites and Symbiodinium *diversity*

Consistent with other studies (Schoenberg and Trench, 1980; Colley, 1984; Goulet and Coffroth, 1997, 2003; Baillie *et al.*, 1998, 2000; Belda-Baillie *et al.*, 1999), our microsatellite data suggest an enormous amount of genotypic diversity within *Symbiodinium*, as illustrated by the following example. At loci CA4.86 and CA6.38, a total of 10 and 12 unique alleles, respectively, were recovered from samples belonging to *Symbiodinium* B184. Pairing alleles from each locus, under the assumption that there are no restrictions against particular combinations of alleles, generates 120 unique genotypes of *Symbiodinium* B184. However, we feel that this is a conservative estimate for several reasons. First, a minimal number of microsatellite loci are being employed. Data from other polymorphic microsatellite loci would distinguish more genotypes within *Symbiodinium* B184. Second, some alleles for CA4.86 and CA6.38 are missing from the data set because they have not yet been sampled (Fig. 1). The inclusion of any of these alleles would generate up to 210 unique genotypes of *Symbiodinium* B184. Third, the *Symbiodinium* B184 populations of hosts such as *P. kuna* possess "null" alleles at these loci, suggesting an additional level of diversity within the group (see above). Last it is extremely unlikely that this high level of genotypic diversity is confined to *Symbiodinium* B184. Thus, microsatellites will doubtlessly uncover high levels of genotypic diversity in most, if not all, of the 16 *Symbiodinium* lineages recognized in cp23S-rDNA phylogenies (Santos *et al.*, 2002, 2003).

Evidence for recombination in Symbiodinium

The finding that vegetative cells of *Symbiodinium* possess a haploid nuclear phase does not preclude recombination within the life cycle of these symbiotic dinoflagellates. For example, other haploid organisms maintain some form of recombination during their life cycle, including the green alga *Chlamydomonas* and members of the Acrasiomycota (cellular slime molds), the Bryophyta (mosses), the Pterophyta (ferns), the Apicomplexa, and the Dinophyceae (dinoflagellates) (Pfiester and Anderson, 1987; Campbell, 1993). In fact, the high allelic variability observed for allozymes (Schoenberg and Trench, 1980; Baillie *et al.*, 1998; Belda-Baillie *et al.*, 1999), random-amplified-polymorphic DNA (RAPDs) (Belda-Baillie *et al.*, 1999; Baillie *et al.*, 2000), and DNA fingerprints (Goulet and Coffroth, 1997, 2003) suggests extensive recombination in *Symbiodinium* (Baillie *et al.*, 2000; reviewed in LaJeunesse, 2001). This evidence for recombination, taken together with our finding of haploidy, lends strong support to *Symbiodinium* life cycle (a), as proposed by Fitt and Trench (1983). However, questions pertaining to recombination in these enigmatic dinoflagellates, such as the factors that induce it and whether it occurs inside or outside a host, remain to be answered.

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